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In Vitro Mutational Analysis of *cis*-Acting RNA Translational Elements within the Poliovirus Type 2 5′ Untranslated Region

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Initiation of translation on poliovirus RNA occurs by internal binding of ribosomes to a region within the 5′ untranslated region (UTR) of the mRNA. This region has been previously roughly mapped between nucleotides 140 and 631 of the 5′ UTR and termed the ribosome landing pad. To identify *cis*-acting elements in the 5′ UTR of poliovirus type 2 (Lansing strain) RNA that confer cap-independent internal initiation, we determined the in vitro translational efficiencies of a series of deletion and point mutations within the 5′ UTR of the mRNA. The results demonstrate that the 3′ border of the core poliovirus ribosome landing pad is located between nucleotides 556 and 585, whereas a region extending between nucleotides 585 and 612 confers enhanced translation. We studied two *cis*-acting elements within this region of the 5′ UTR: a pyrimidine stretch which is critical for translation and an AUG (number 7 from the 5′ end) that is located ~20 nucleotides downstream from the pyrimidine stretch and augments translation. We also show that the stem-loop structure which contains this AUG is not required for translation.

Poliovirus mRNA, like other picornavirus mRNAs, is unusual in that unlike eukaryotic cellular mRNAs, it is not capped at its 5′ end but rather terminates with a monophosphorylated uridine (11, 29). In addition, the 5′ untranslated regions (UTRs) of picornavirus mRNAs contain a high number of AUG codons (e.g., 10 for encephalomyocarditis virus [EMCV] and 6 to 8 for poliovirus, depending on the serotype). These observations and the finding that translation of poliovirus RNA is cap independent (31) and occurs in vivo and in vitro under conditions in which cap-mediated translation is impaired (for a review, see reference 26) suggested that initiation of translation on poliovirus RNA is very different from that of cellular mRNAs.

Initiation of translation on poliovirus mRNA occurs by direct binding of ribosomes to an internal sequence in the 5′ UTR which has been termed the ribosome landing pad (33). The region responsible for cap-independent translation was found to be quite large, comprising nucleotides 140 to 631 (31). When fused to heterologous mRNAs, this element could function in *cis* to render translation cap independent (31, 45). Analysis of insertions and deletions introduced into the 5′ UTR of the Mahoney (4, 8, 44) or Sabin (20) strain of poliovirus type 1 has confirmed the importance of the cap-independent region for viral translation in vivo and in vitro.

Internal initiation of translation has been shown conclusively for other picornaviruses, such as EMCV (15, 16), and is strongly suggested for foot-and-mouth disease virus (FMDV) (21) and rhinovirus (2). The picornavirus family can be divided into two classes based on comparison of the secondary structures of their mRNA 5′ UTRs. Secondary structure predictions of picornavirus RNAs, based on phylogenetic comparisons and enzymatic and chemical probing, revealed two different patterns of conserved structures, one for enteroviruses and rhinoviruses (22, 35, 36, 40) and another for cardioviruses and aphthoviruses (34). These structural differences may also underlie the differences in the translation characteristics between cardiovirus-aphthovirus and enterovirus-rhinovirus RNAs (13). RNAs from EMCV and FMDV are translated with high efficiency in reticulocyte lysates, whereas poliovirus and rhinovirus RNAs are translated poorly in the reticulocyte lysate but translation can be augmented by addition of HeLa cell extracts (2, 9). Not surprisingly, the mechanisms of internal initiation appear to be different in certain aspects for these two classes of RNAs (14).

For poliovirus, internal initiation of translation requires the involvement of *cis*-acting RNA elements (30) and *trans*-acting factors (7, 24, 27). Several features in the poliovirus 5′ UTR are conserved among the different poliovirus serotypes. Of the six to eight upstream AUGs in poliovirus, three are conserved among the three serotypes (AUGs 4, 5, and 7 in type 2; reference 43). To address the importance of the upstream AUGs, each of the adenine nucleotides in the AUGs in poliovirus type 2 were mutagenized to uridines (30). Only mutation of the adenine of AUG 7 (position 588) was shown to have a negative effect on poliovirus translation in vitro and viral growth in vivo (30). AUG 7 is part of a stem structure (number 7 from the 5′ end, encompassing nucleotides 583 to 620) of the 5′ UTR (Fig. 1A) (22, 35, 36, 40). This raises the question of whether the AUG per se or the stem-loop structure is important for efficient translation. Upstream (~20 nucleotides) of this stem-loop structure and in a phylogenetically conserved spacing from AUG 7 is a highly conserved pyrimidine stretch. This pyrimidine stretch also resides in a similarly spaced distance upstream from the translation initiators of FMDV, EMCV, and other cardiovirus RNAs. Deletion and mutational analysis of this stretch in EMCV and FMDV demonstrated that it constitutes an essential domain for translation (17, 21).

The mechanism of internal initiation of translation on picornavirus RNAs is poorly understood. The finding that a large segment of the 5′ UTR is required for internal initiation of translation suggests that tertiary structure is likely to play a key role in ribosome binding. The three-dimensional structure of the 5′ UTR has not been determined. It is important, however, first to define *cis*-acting regions on the

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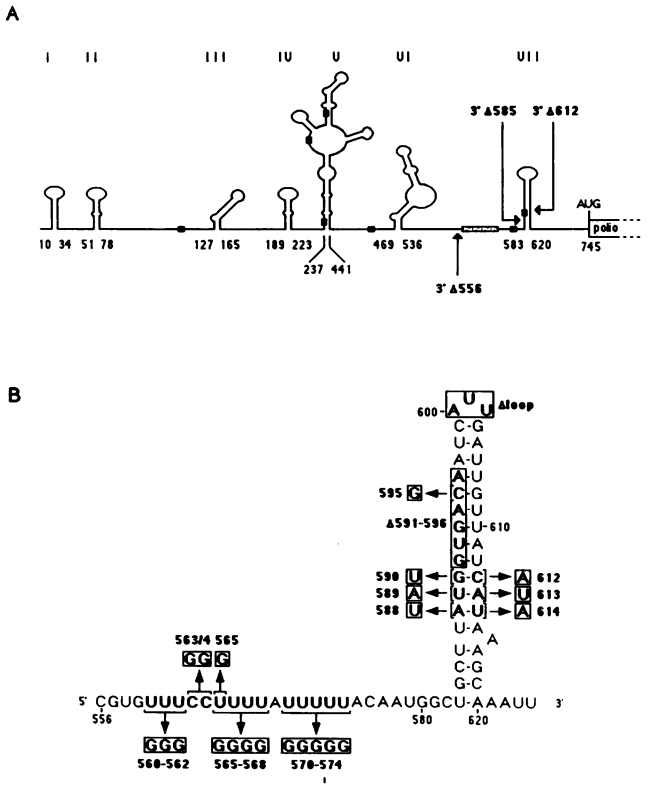

FIG. 1. Map of wild-type and mutant poliovirus type 2 (Lansing) mRNAs. (A) Schematic map of the secondary structure of the 5′ UTR. The map was adapted from Skinner et al. (40) and is not to scale. The nucleotide positions for the start and end of the stem-loop structures are indicated. Symbols: ■, upstream AUGs; ⟹, pyrimidine stretch spanning nucleotides 560 to 574; →, endpoints of 3′ deletions. (B) Nucleotide sequence of the pyrimidine stretch, stem-loop VII, and the mutations generated by site-directed mutagenesis. Mutations are boxed and referred to by their nucleotide positions, except for Δloop, which represents a deletion of nucleotides 600 to 602.

poliovirus RNA that are involved in efficient internal initiation.

MATERIALS AND METHODS

Construction of plasmids. The construction of poliovirus chloramphenicol acetyltransferase (CAT) plasmid P2CAT in the KS vector was as follows. The CAT open reading frame was isolated from pSV2CAT (31) by restriction with *Ban*I, repair with the Klenow fragment, and ligation to *Hin*dIII synthetic linkers, followed by restriction with *Hin*dIII. The insert was purified from a low-melting-point agarose gel and ligated into the Bluescript KS+ vector (Stratagene) at the *Hin*dIII site to create KSCAT. A *Pst*I-*Eco*RV restriction fragment (from pP2-5′) containing nucleotides 1 to 730 of the poliovirus type 2 Lansing 5′ noncoding region (30) was made blunt by digestion with T4 DNA polymerase and then inserted into the *Eco*RV site of KSCAT to generate KSP2CAT. 3′ deletions were introduced in the poliovirus 5′ UTR in KSP2RI, which is described in reference 28. The resulting deletions were removed from KSP2RI by restriction with *Sac*I and *Pst*I, made blunt by T4 DNA polymerase, and inserted into the *Eco*RV site of KSCAT. Mutations in the pyrimidine stretch were created and subcloned as previously described (28). An *Eco*RI fragment containing the mutant DNAs from plasmid pSVGHP2CAT was isolated and substituted for an *Eco*RI fragment in KSP2CAT.

The starting plasmid used to introduce stem-loop mutants was a *Hin*dIII-*Eco*RI restriction fragment from pP2-5′ inserted into *Hin*dIII-*Eco*RI-digested M13mp19. Point mutations were made by the two-primer method (46). Following mutagenesis, the DNA was removed from M13 and subcloned in pSP64CAT as previously described (30). All mutations were confirmed by sequencing by using the dideoxy chain termination method (38) before and after subcloning in pKS and pSP64. In all constructs, the initiator AUG of CAT is used to initiate CAT protein synthesis.

In vitro transcriptions. Plasmids were linearized with *Cla*I (for KSP2CAT deletion and pyrimidine mutants) or *Bam*HI (for SP6P2CAT stem-loop mutants) and used as templates for transcription by T7 or SP6 RNA polymerase, respectively, as previously described (32), except that the GTP and cap analog (m^7 GpppG) concentrations were 50 and 500 μM, respectively. mRNA was labeled with [5-^{3}H]CTP and purified as previously described (32). The specific activity of the mRNA was calculated as 1.4×10^5 cpm/μg. A portion of each transcription (i.e., the amount used to program in vitro translation extracts) was analyzed on 0.7% formaldehyde agarose gels. Gels were treated with En^{3}Hance (NEN Corp. Inc.) and exposed against X-ray film at −70°C.

In vitro translations. In vitro translations of the various CAT mRNAs were performed in HeLa cell extracts (37). The translations were performed with 12.5-μl reaction mixtures containing 0.2 μg of various mRNAs. Incubations took place for 60 min at 37°C. Translation products were labeled with [^{35}S]methionine and analyzed by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis. Gels were fixed in 40% methanol–7.5% acetic acid, treated with En^{3}Hance, and exposed against X-ray film at −70°C. Translations for each experiment were performed at least three times with different mRNA preparations and several mRNA concentrations. Quantitation of translation efficiencies was performed by scanning X-ray films with an LKB soft-laser densitometer.

RESULTS

Translation of poliovirus 5′ UTR 3′ deletions. A series of plasmids was constructed in which deletions were introduced into the poliovirus 5′ UTR (P2) and placed in front of the coding sequence for the bacterial enzyme CAT. The P2CAT sequences were placed downstream of the T7 RNA polymerase promoter. RNA produced by transcription of these plasmids was used to program in vitro HeLa cell translation extracts. Translations were performed with both unmethylated and methylated capped mRNAs. The results obtained were identical for both sets of constructs, and only those for the capped methylated mRNAs are shown in this report. The integrity of the RNAs used in these experiments was analyzed by electrophoresis on formaldehyde agarose gels (data not shown). The stability of the RNAs during the translation reaction, under the conditions described in this study, has previously been investigated (31). While the mRNA is degraded during incubation under translation conditions, the relative stability between mRNAs does not vary (31; data not shown). Thus, the differences in translational efficiency among the different P2CAT mutants cannot be attributed to differential mRNA stabilities.

The effect of 3′ deletions on translation is shown in Fig. 2. A 3′ deletion extending to nucleotide 612 (3′ Δ612; Fig. 1A)

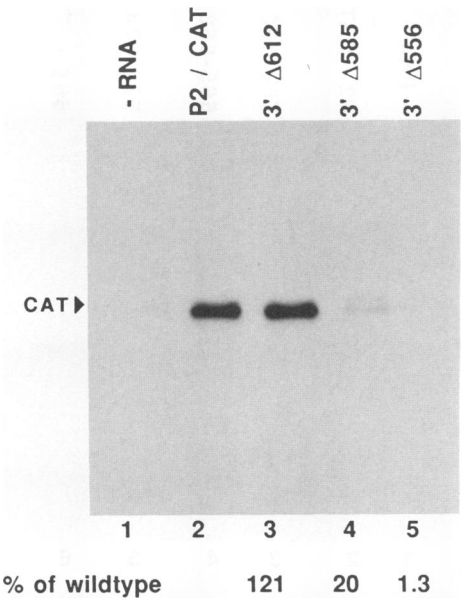

FIG. 2. Translation of capped P2CAT 3′ deletion mRNAs in HeLa cell extracts. Translations were performed with 200 ng of mRNA in 12.5 μl reactions and processed as described in Materials and Methods.

did not decrease the translational efficiency of P2CAT mRNA compared with the full-length 5′ UTR of poliovirus mRNA (Fig. 2, compare lanes 3 and 2). This deletion removes part of the right side of stem-loop VII (Fig. 1A) and suggests that the integrity of stem-loop VII is not important for translation (see below). Deletion to nucleotide 585 (3′ Δ585) resulted in significant (fivefold) inhibition of CAT mRNA translation (compare lanes 4 and 2). This suggests that sequences between nucleotides 585 and 612 are important for efficient translation. This deletion removes all of stem-loop VII and the conserved upstream AUG 7 comprising nucleotides 588 to 590 (Fig. 1). Results described below more precisely address the effects of these features on translation. A further 3′ deletion extending to nucleotide 556 (3′ Δ556) abolished translation (Fig. 2, compare lanes 5 and 2). Thus, a deletion between nucleotides 556 and 585 removes sequences critical for translation from the poliovirus 5′ UTR. In this region lie AUG 6 (which is not conserved among poliovirus serotypes) and a pyrimidine stretch which is highly conserved among picornaviruses (Fig. 1A). Mutation of the A nucleotide of AUG 6 was previously shown to have no effect on the translation from the poliovirus 5′ UTR (30), while point mutations in the pyrimidine stretch have dramatic effects on translation (see below).

In the accompanying report (28), the effects of these deletions on translation were assayed in vivo after transfection of bicistronic constructs. The results obtained with constructs 3′ Δ612, 3′ Δ585, and 3′ Δ556 were very similar to the in vitro results and together demonstrate that the 3′ border of the core internal ribosome-binding site lies between nucleotides 556 and 585.

Translation of RNAs mutated in stem-loop VII. In view of the above results, we asked whether the stem-loop structure or the primary sequence of stem-loop VII is the important determinant in effecting efficient poliovirus RNA translation. The stem-loop structure includes AUG 7. It has previously been shown that the adenine nucleotide in AUG 7 affects the translational efficiency of poliovirus mRNA (30). To determine whether this effect was due to mutation of the upstream AUG or disruption of base pairing in the stem, we changed the U (589) and G (590) nucleotides of AUG 7 to A and U, respectively (Fig. 1B). In addition, the nucleotides on the right side of the stem predicted to base pair to AUG 7 were mutated. The mutations chosen are compensatory to those made for the AUG on the left side of the stem (Fig. 1B). The translation of the corresponding capped P2CAT mRNAs in HeLa cell extracts is shown in Fig. 3. Mutations of the AUG 7 nucleotides on the left side of the stem all resulted in a threefold decrease in translation of CAT mRNA (Fig. 3, compare lanes 3, 6, and 9 with lane 2). In contrast, translation of mRNAs with nucleotide substitutions on the opposite (right) side of the stem was as good as that of wild-type mRNA (compare lanes 4, 7, and 10 with lane 2). These results corroborate the observation that a 3′ deletion to

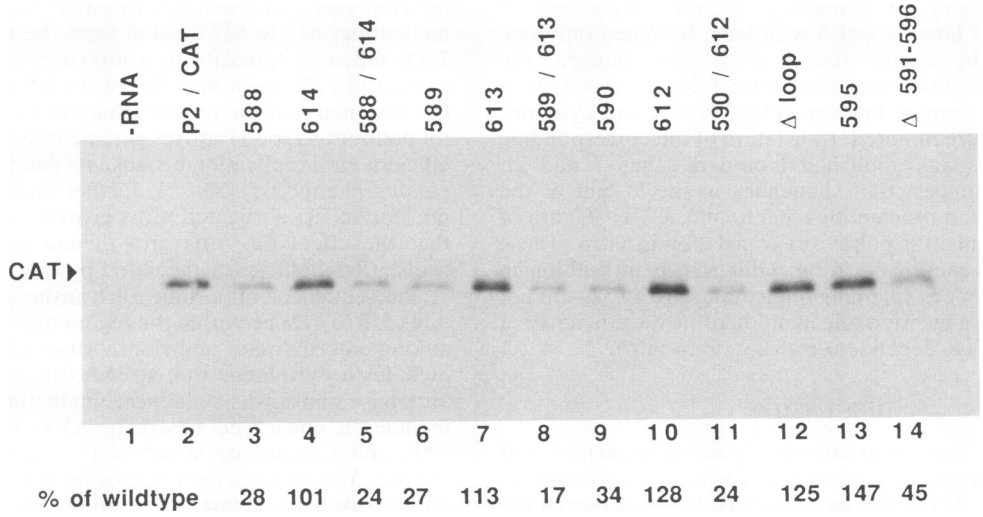

FIG. 3. Translation of capped P2CAT mRNAs containing stem-loop mutations in HeLa cell extracts. Translations were performed with 200 ng of mRNA in 12.5-μl reactions and processed as described in Materials and Methods.

nucleotide 612 had no effect on the translational efficiency of P2CAT mRNA but deletion to nucleotide 585 reduced translation (Fig. 2). We then combined the compensatory mutations to restore base pairing. Translation of the corresponding mRNAs in a HeLa cell extract was reduced (four to fivefold) compared with the wild type, as was observed with the RNAs containing single mutations in AUG 7 on the left side of the stem (compare lanes 5, 8, and 11 with lane 2). These results demonstrate, in accordance with previous findings (30), that intact base pairing between AUG 7 and complementary nucleotides is not required for in vitro translation. Furthermore, deletion of the loop sequences and a point mutation at nucleotide 595, which is expected to destabilize the stem loop structure, had no effect on translation in vitro (lanes 12 and 13). Because point mutations might not fully disrupt the integrity of a 16-bp stem-loop structure, we introduced a deletion of six nucleotides (Δ591-596) in the stem of stem-loop structure VII. Translation of CAT mRNA derived from this construct was decreased by only twofold compared with that of the wild type (compare lanes 14 and 2). Taken together, these results suggest that the integrity of stem-loop VII does not play a role in internal ribosome binding. Clearly, sequences between nucleotides 585 and 612 are important but not critical for translation. The augmenting role for AUG 7 in translation of the poliovirus 5′ UTR was also demonstrated in vivo in a bicistronic construct (28).

Translation of RNAs mutated in the pyrimidine stretch. Deletion analysis of the 3′ end of the poliovirus 5′ UTR revealed that the region spanning nucleotides 556 to 585 is critical for translation in vitro (Fig. 2) and in vivo (28). This deletion removes a pyrimidine stretch (particularly uridine rich) that is highly conserved among picornaviruses and was shown before to be important for translation of FMDV and EMCV (17, 21). To determine the specific sequences that play a role in poliovirus RNA translation, we used site-directed mutagenesis to change nucleotides in this region. The mutations chosen are shown in Fig. 1B, and the translation of the corresponding capped P2CAT mRNAs in HeLa cell extracts is shown in Fig. 4. Mutation of the three 5′ uridine residues to guanosines (560 to 562) dramatically reduced translation of P2CAT mRNA (Fig. 4, compare lanes 3 and 2). Nucleotide substitutions of the middle (565 to 568) and 3′ (570 to 574) stretches of uridines by guanosines reduced translation approximately 10- and 40-fold, respectively (compare lanes 4 and 5 with lane 2). When only one uridine from the middle stretch (565) was changed, the translational efficiency was about threefold less than that of the wild type (compare lanes 6 and 2). When both cytidines (563 and 564) were mutated, translation of the corresponding P2CAT mRNA was abolished (compare lanes 7 and 2). These results suggest that sequences in the 5′ half of the pyrimidine stretch (nucleotides 560 to 565; UUUCC) are of critical importance for poliovirus translation in vitro. These mutations also caused a strong reduction in translation in vivo (28). Sequences spanning nucleotides 565 to 574 are not critical but play a positive role in modulating the efficiency of poliovirus 5′ UTR-dependent translation in vitro.

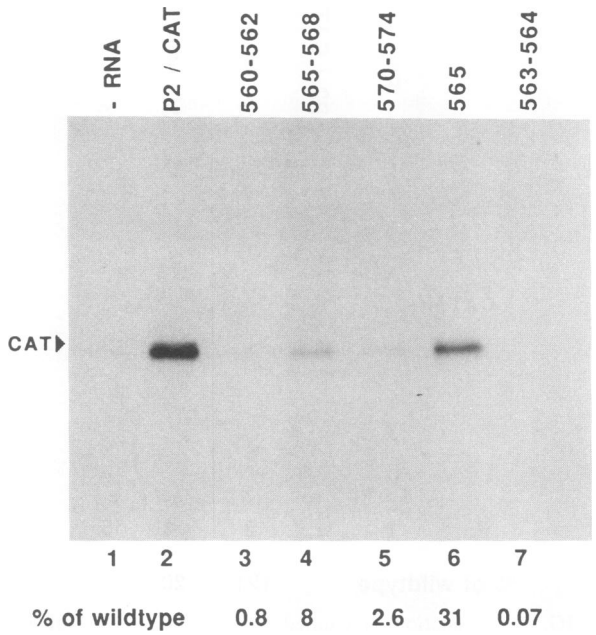

FIG. 4. Translation of capped P2CAT mRNAs containing pyrimidine mutations in HeLa cell extracts. Translations were performed with 200 ng of mRNA in 12.5-μl reactions and processed as described in Materials and Methods.

DISCUSSION

The results in this report identify *cis*-acting sequences that are required for efficient internal initiation of translation in the poliovirus 5′ UTR. We have mapped the 3′ border of the core ribosome landing pad between nucleotides 556 and 585 (within a pyrimidine stretch) and an extended ribosome landing pad with a 3′ border between nucleotides 585 and 612. This conclusion is based on the finding that a 3′ deletion to nucleotide 612 had no effect on translation. A further 3′ deletion to nucleotide 585 reduced translation, but deletion to nucleotide 556 abolished translation (Fig. 2). Thus, sequences spanning the region between nucleotides 556 and 585 are critical for translation and sequences extending to nucleotide 612 have a positive effect on poliovirus translation.

The results presented here support and extend previous observations. A 3′ deletion in the poliovirus 5′ UTR extending to nucleotide 631 had no effect on translation of P2CAT mRNA. However, when the deletion extended to nucleotide 461, translation was abolished in poliovirus and mock-infected HeLa cell extracts in vitro (31). A transcript with nucleotides 517 to 627 deleted from the poliovirus type 1 5′ UTR failed to translate in a mixed reticulocyte-HeLa cell extract (4). Deletion analysis of an infectious cDNA clone has revealed that the region spanning nucleotides 564 to 599 (of poliovirus type 1) carries genetic information required for efficient viral replication because its deletion caused a small-plaque phenotype (20). A further deletion extending to nucleotide 561 abrogated virus growth (12). It is very likely that the effect on virus growth can be attributed to the translational efficiency mediated by the 5′ UTR.

The sequences important for translation in vitro (nucleotides 556 to 612) lie within the region that is highly conserved among enteroviruses and rhinoviruses (20, 43). This region includes a pyrimidine-rich stretch (nucleotides 560 to 574), an 8-base consensus sequence (nucleotides 583 to 590) that includes a conserved upstream AUG (nucleotides 588 to 590), and a stem-loop structure (~−12 kcal [1 cal = 4.184 J]/mol). We asked whether specific primary nucleotide sequences or a secondary structure in this region is important for poliovirus translation. RNAs harboring point mutations within the AUG triplet at nucleotides 588 to 590 were

translated with reduced efficiency (Fig. 3). Nucleotide substitutions on the opposite side of the stem had no effect, whereas the double mutants had reduced translational efficiency. We conclude that the primary nucleotide sequence encompassing AUG 7, and not the stem-loop structure in which it resides, is an important feature for modulation of poliovirus translation. It is possible that AUG 7 is recognized as an initiation codon if ribosomes land just upstream of AUG 7. We tested this possibility by a frameshift mutation at position 647 in the AUG 7 open reading frame. This mutation results in an open reading frame that terminates within the CAT-coding region and encodes an 11,500-Da polypeptide. Translation of this open reading frame would shunt ribosomes past the initiator codon in CAT. Following translation in a HeLa cell extract, we could not detect an 11,500-Da polypeptide, nor was the translational efficiency of CAT altered (25). These results suggest that if AUG 7 serves as an initiation codon it is a very weak one, as previously suggested by Jackson et al. for poliovirus type 1 (14).

The sequences essential for translation in vitro (nucleotides 556 to 585) comprise a pyrimidine stretch that is present in all picornaviruses. We have demonstrated by point mutagenesis that only the 5′ half of the pyrimidine stretch, nucleotides 560 to 564 (UUUCC), is critical for poliovirus translation in vitro and in vivo (28). Uridine residues in the middle and 3′ half, while not critical, nevertheless play a stimulatory role in ribosome landing pad function. Translation of RNAs harboring mutations in the uridine residues in the middle of the pyrimidine stretch is reduced to a much greater extent in vitro than in vivo (28). This may reflect the fact that factors important for efficient translation are limiting in cell extracts. Our results demonstrate that the conserved pyrimidine stretch in poliovirus is functionally equivalent to that of FMDV and EMCV. The 3′ border of the internal ribosomal entry site in the EMCV 5′ UTR has been mapped within the pyrimidine stretch (17). Deletion of this pyrimidine stretch in EMCV and point mutations in this stretch in FMDV dramatically decreased the translational efficiency in vitro and in vivo (17, 21).

Another conserved feature that could be important for poliovirus translation is the spacing between the pyrimidine stretch and AUG 7. The distance between the UUUCC motif and a conserved AUG among poliovirus serotypes is 22 or 23 nucleotides (Fig. 1; reference 28). Similarly, approximately 20 nucleotides separate the UUUCC motif in the pyrimidine stretch and an AUG in rhinoviruses, cardioviruses, and aphthoviruses. The significance of this feature was not experimentally tested in this study, but it was shown to be important (1). Several functions for this pyrimidine stretch-AUG configuration that are consistent with internal ribosome binding can be envisaged. It is conceivable that proteins interact with different elements of the 5′ UTR to facilitate ribosome binding. A search for such proteins using gel electrophoresis mobility shift and UV-induced cross-linking assays yielded several proteins that interact with various regions of the 5′ UTR (7, 24, 27). One factor (p52) has been shown to bind to an RNA fragment encompassing nucleotides 559 to 624 (24). This fragment includes the pyrimidine stretch and AUG 7. In a more extensive study, we have found that p52 can bind to RNA fragments comprising nucleotides 539 to 646 but not to sequences upstream or downstream from this region (25). Several protein-RNA complexes with 5′ UTR RNA fragments encompassing nucleotides 98 to 182 and 510 to 629 were reported (7). These complexes contained eukaryotic initiation factor 2. However, in both studies sequence-specific interactions were not identified. Eukaryotic initiation factor 2 has been proposed to bind RNA (18), although its specificity of interaction is moot. Another region of the 5′ UTR containing nucleotides 178 to 224 (in poliovirus type 1) interacts specifically with a 50-kDa polypeptide (27). The UV cross-linking technique has been used to detect cellular proteins, p57 and p58, that interact with the 5′ UTR of EMCV (5, 17) and FMDV (23). These proteins have been shown to bind to RNA sequences that are functionally important for translation. Interaction of these proteins with the pyrimidine stretch has not been reported.

Another possible function of the pyrimidine stretch in translation initiation is analogous to the Shine-Dalgarno sequence in the prokaryotic system, as has previously been suggested (3). The pyrimidine stretch shows sequence complementarity to purine-rich stretches in mammalian 18S rRNA (1, 3, 17, 28). In particular, nucleotides 562 to 568 (5′-UUCCUUU-3′) of poliovirus (type 2) mRNA could theoretically base pair to nucleotides 1194 to 1200 (3′-AAAG GAA-5′) of human 18S rRNA (28). The sequence AAAG GAA in the putative binding site is conserved in all 18S rRNAs (6).

A molecular mechanism for internal ribosome binding on poliovirus RNA is beginning to emerge. This mechanism suggests that ribosomes bind internally to the 5′ UTR in the vicinity of the pyrimidine stretch. However, the identification of a large sequence in the poliovirus 5′ UTR that is required for internal initiation of translation (31, 44) argues strongly for the involvement of a superstructure in this process. It is predicted from the secondary structure models that the region surrounding the pyrimidine stretch is unstructured. Thus, 40S ribosomes could interact with this region via 18S rRNA or *trans*-acting factors within the constraints of a global superstructure. Earlier studies support the hypothesis that ribosomes bind to this region and then translocate to the initiator AUG (19, 33). Insertion of a secondary structure downstream of AUG 7 drastically reduced translation in an in vitro translation system (33). Also, insertion of a sequence containing an AUG codon downstream from the AUG 7 equivalent (at position 702) in poliovirus type 1, Mahoney strain, generated a virus exhibiting a small-plaque phenotype. Wild-type revertants retained the inserted sequence, but the AUG was either mutated or deleted (19).

Translation plays a significant role in poliovirus neurovirulence, as demonstrated with a point mutation at nucleotide 472 that attenuates poliovirus type 3 neurovirulence in vivo (10). This substitution results in decreased translation (41, 42). Thus, identification of mutations in the 5′ UTR that alter mRNA translation reveals sequences that are important for virus growth and neurovirulence. Future work on the analysis of *cis*- and *trans*-acting elements that are involved in poliovirus and other picornavirus translation should also be applicable to understanding of the translation of cellular mRNAs. It is becoming clear that a group of cellular mRNAs uses a similar mechanism to initiate translation (39). Furthermore, it is likely that internal initiation of translation could play a significant role in regulating protein synthesis in eukaryotes.

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REFERENCES

1. **Agol, V. I.** 1991. Personal communication.
2. **AlSaadi, S., S. Hassard, and G. Stanway.** 1989. Sequences in the 5′ noncoding region of human rhinovirus type 14 RNA that affect *in vitro* translation. J. Gen. Virol. **70**:2799–2804.
3. **Beck, E., S. Forss, K. Strebel, R. Cattaneo, and G. Feil.** 1983. Structure of the FMDV translation initiation site and of the structural protein. Nucleic Acids Res. **11**:7873–7885.
4. **Bienkowska-Szewczyk, K., and E. Ehrenfeld.** 1988. An internal 5′-noncoding region required for translation of poliovirus RNA in vitro. J. Virol. **62**:3068–3072.
5. **Borovjagin, A. V., A. G. Evstafieva, T. Y. Ungarova, and I. N. Shatsky.** 1990. A factor that specifically binds to the 5′-untranslated region of encephalomyocarditis virus RNA. FEBS Lett. **261**:237–240.
6. **Chan, Y.-L., R. Gutell, H. F. Noller, and I. G. Wool.** 1984. The nucleotide sequence of a rat 18S ribosomal ribonucleic acid gene and a proposal for the secondary structure of 18S ribosomal ribonucleic acid. J. Biol. Chem. **259**:224–230.
7. **del Angel, R. M., A. G. Papavassiliou, C. Fernandez-Tomas, S. J. Silverstein, and V. R. Racaniello.** 1989. Cell proteins bind to multiple sites within the 5′ untranslated region of poliovirus RNA. Proc. Natl. Acad. Sci. USA **86**:8299–8303.
8. **Dildine, S. L., and B. L. Semler.** 1989. The deletion of 41 proximal nucleotides reverts a poliovirus mutant containing a temperature-sensitive lesion in the 5′ noncoding region of genomic RNA. J. Virol. **63**:847–862.
9. **Dorner, A. J., B. L. Semler, R. J. Jackson, R. Hanecak, E. Duprey, and E. Wimmer.** 1984. In vitro translation of poliovirus RNA: utilization of internal initiation sites in reticulocyte lysate. J. Virol. **50**:507–514.
10. **Evans, D. M. A., G. Dunn, P. D. Minor, G. C. Schild, A. J. Cann, G. Stanway, J. W. Almond, K. Currey, and J. V. Maizel, Jr.** 1985. Increased neurovirulence associated with a single nucleotide change in a noncoding region of the Sabin type 3 poliovaccine genome. Nature (London) **314**:548–550.
11. **Hewlett, M. J., J. K. Rose, and D. Baltimore.** 1976. 5′-terminal structure of poliovirus polyribosomal RNA is pUp. Proc. Natl. Acad. Sci. USA **73**:327–330.
12. **Iizuka, N., M. Kohara, K. Hagino-Yamagishi, S. Abe, T. Komatsu, K. Tago, M. Arita, and A. Nomoto.** 1989. Construction of less neurovirulent polioviruses by introducing deletions into the 5′ noncoding sequence of the genome. J. Virol. **63**:5354–5363.
13. **Jackson, R. J.** 1989. Comparison of encephalomyocarditis virus and poliovirus with respect to translation initiation and processing in vitro, p. 51–71. *In* B. L. Semler and E. Ehrenfeld (ed.), Molecular aspects of picornavirus infection and detection. American Society for Microbiology, Washington, D.C.
14. **Jackson, R. J., M. T. Howell, and A. Kaminski.** 1990. The novel mechanism of initiation of picornavirus RNA translation. Trends Biochem. Sci. **15**:477–483.
15. **Jang, S. K., M. V. Davies, R. J. Kaufman, and E. Wimmer.** 1989. Initiation of protein synthesis by internal entry of ribosomes into the 5′ nontranslated region of encephalomyocarditis virus RNA in vivo. J. Virol. **63**:1651–1660.
16. **Jang, S. K., H.-G. Kräusslich, M. J. H. Nicklin, G. M. Duke, A. C. Palmenberg, and E. Wimmer.** 1988. A segment of the 5′ nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. J. Virol. **62**:2636–2643.
17. **Jang, S. K., and E. Wimmer.** 1990. Cap-independent translation of encephalomyocarditis virus RNA: structural elements of the internal ribosomal entry site and involvement of a cellular 57-kD RNA binding protein. Genes Dev. **4**:1560–1572.
18. **Kaempfer, R., J. van Emmelo, and W. Fiers.** 1981. Specific binding of eukaryotic initiation factor 2 to satellite tobacco necrosis virus RNA at a 5′-terminal sequence comprising the ribosome binding site. Proc. Natl. Acad. Sci. USA **78**:1542–1546.
19. **Kuge, S., N. Kawamura, and A. Nomoto.** 1989. Genetic variation occurring on the genome of an in vitro insertion mutant of poliovirus type 1. J. Virol. **63**:1069–1075.
20. **Kuge, S., and A. Nomoto.** 1987. Construction of viable deletion and insertion mutants of the Sabin strain of type 1 poliovirus: function of the 5′ noncoding sequence in viral replication. J. Virol. **61**:1478–1487.
21. **Kühn, R., N. Luz, and E. Beck.** 1990. Functional analysis of the internal translation initiation site of foot-and-mouth disease virus. J. Virol. **64**:4625–4631.
22. **Le, S.-Y., and M. Zuker.** 1990. Common structures of the 5′ non-coding RNA in enteroviruses and rhinoviruses. Thermodynamical stability and statistical significance. J. Mol. Biol. **216**: 729–741.
23. **Luz, N., and E. Beck.** 1990. A cellular 57 kDa protein binds to two regions of the internal translation initiation site of foot-and-mouth disease virus. FEBS Lett. **269**:311–314.
24. **Meerovitch, K., J. Pelletier, and N. Sonenberg.** 1989. A cellular protein that binds to the 5′-noncoding region of poliovirus RNA: implications for internal translation initiation. Genes Dev. **3**:1026–1034.
25. **Meerovitch, K., and N. Sonenberg.** Unpublished data.
26. **Meerovitch, K., N. Sonenberg, and J. Pelletier.** 1991. The translation of picornaviruses, p. 273–292. *In* H. Trachsel (ed.), Translation in eukaryotes. C.R.C. Press, Inc., Boca Raton, Fla.
27. **Najita, L., and P. Sarnow.** 1990. Interaction of a cellular 50kDa protein with an RNA hairpin in the 5′ noncoding region of the poliovirus genome: evidence for a transient covalent RNA-protein bond. Proc. Natl. Acad. Sci. USA **87**:5846–5850.
28. **Nicholson, R., J. Pelletier, S.-Y. Le, and N. Sonenberg.** 1991. Structural and functional analysis of the ribosome landing pad of poliovirus type 2: in vivo translation studies. J. Virol. **65**:5886–5894.
29. **Nomoto, A., Y. F. Lee, and E. Wimmer.** 1976. The 5′ end of poliovirus mRNA is not capped with $m^7G(5')ppp(5')Np$. Proc. Natl. Acad. Sci. USA **73**:375–380.
30. **Pelletier, J., M. E. Flynn, G. Kaplan, V. Racaniello, and N. Sonenberg.** 1988. Mutational analysis of upstream AUG codons of poliovirus RNA. J. Virol. **62**:4486–4492.
31. **Pelletier, J., G. Kaplan, V. R. Racaniello, and N. Sonenberg.** 1988. Cap-independent translation of poliovirus mRNA is conferred by sequence elements within the 5′ noncoding region. Mol. Cell. Biol. **8**:1103–1112.
32. **Pelletier, J., and N. Sonenberg.** 1985. Insertion mutagenesis to increase secondary structure within the 5′ noncoding region of a eukaryotic mRNA reduces translational efficiency. Cell **40**:515–526.
33. **Pelletier, J., and N. Sonenberg.** 1988. Internal initiation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature (London) **334**:320–325.
34. **Pilipenko, E. V., V. M. Blinov, B. K. Chernov, T. M. Dmitrieva, and V. I. Agol.** 1989. Conservation of the secondary structure elements of the 5′-untranslated region of cardio- and aphthovirus RNAs. Nucleic Acids Res. **17**:5701–5711.
35. **Pilipenko, E. V., V. M. Blinov, L. I. Romanova, A. N. Sinyakov, S. V. Maslova, and V. I. Agol.** 1989. Conserved structural domains in the 5′-untranslated region of picornaviral genomes: an analysis of the segment controlling translation and neurovirulence. Virology **168**:201–209.
36. **Rivera, V. M., J. D. Welsh, and J. V. Maizel, Jr.** 1988. Comparative sequence analysis of the 5′ noncoding region of enteroviruses and rhinoviruses. Virology **165**:42–50.
37. **Rose, J. K., H. Trachsel, K. Leong, and D. Baltimore.** 1978. Inhibition of translation by poliovirus: inactivation of a specific initiation factor. Proc. Natl. Acad. Sci. USA **75**:2732–2736.
38. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
39. **Sarnow, P.** 1991. Personal communication.
40. **Skinner, M. A., V. R. Racaniello, G. Dunn, J. Cooper, P. D. Minor, and J. W. Almond.** 1989. New model for the secondary

structure of the 5′ non-coding RNA of poliovirus is supported by biochemical and genetic data that also show that RNA secondary structure is important in neurovirulence. J. Mol. Biol. **207**:379–392.

41. **Svitkin, Y. V., N. Cammack, P. D. Minor, and J. W. Almond.** 1990. Translation deficiency of the Sabin type 3 poliovirus genome: association with an attenuating mutation C_{472}-U. Virology **175**:103–109.
42. **Svitkin, Y. V., S. V. Maslova, and V. I. Agol.** 1985. The genomes of attenuated and virulent poliovirus strains differ in their *in vitro* translation efficiencies. Virology **147**:243–252.
43. **Toyoda, H., M. Kohara, Y. Kataoka, T. Suganuma, T. Omata, N. Imura, and A. Nomoto.** 1984. Complete nucleotide sequences of all three poliovirus serotype genomes. Implications for genetic relationship, gene function and antigenic determinants. J. Mol. Biol. **174**:561–585.
44. **Trono, D., R. Andino, and D. Baltimore.** 1988. An RNA sequence of hundreds of nucleotides at the 5′ end of poliovirus RNA is involved in allowing viral protein synthesis. J. Virol. **62**:2291–2299.
45. **Trono, D., J. Pelletier, N. Sonenberg, and D. Baltimore.** 1988. Translation in mammalian cells of a gene linked to the poliovirus 5′ noncoding region. Science **241**:445–448.
46. **Zoller, M. J., and M. Smith.** 1982. Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment of DNA. Nucleic Acids Res. **10**:6487–6500.